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Extremophilic Enzymatic Response: Role of Proteins in Controlling Selenium Nanoparticle Synthesis

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14. ABSTRACT

We have studied a thermophilic bacteria GWE1 belonging to Geobacillus genus able to biosynthesize Se0 nanoparticles. Elemental analysis and composition of the particles were verified using transmission electron microscopy and energy-dispersive X-ray analysis. The nanoparticles have a defined spherical shape and are composed by Se0. Previous experiments showed that the presence of the whole microorganism for the reduction of selenite was not necessary. We have isolated an intracellular NADPH/NADH-dependent reductase which mediates selenium nanoparticles synthesis under aerobic conditions. The enzyme was purified and identified as a 1-pyrroline-5-carboxylate dehydrogenase by mass spectroscopy MALDI-TOF TOF technique. FT-IR analysis was utilized to characterize the chemical surface of Se nanoparticles. These results strongly suggest that the biosynthesis of Se nanoparticles is mediated by NADH-dependent enzymes.
This ecologically-friendly process allows an efficient control of size, shape and composition of the generated particles. Additionally biosynthesized nanoparticles display good surface bioactivity.

15. SUBJECT TERMS

Extremophiles, biosynthesis of metallic nanoparticles, enzyme mediated process

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Final Performance Report

Title:
Extremophilic Enzymatic Response: Role of Proteins in Controlling Selenium Nanoparticle Synthesis.

Research Interest Category:
Mathematics, Information and Life Sciences (NL), Natural Materials and Systems.

Corresponding Program Manager:
Mr. James Fillerup

Main Objective

To study the role of at least one protein (enzyme) from E1 (GWE1) on the synthesis of nano-Se particles.

Note: This project started in January 2013 and finished in November 2014

Specific Objectives addressed in this Report:

1. Objective: To identify protein(s) or enzyme(s) involved in nanoparticles formation.

To identify the proteins or enzyme(s) involved in nanoparticles formation, a purification process was designed. Since biological selenite reduction seems to be mediated by electron transfer from NADPH/NADH by NADPH/NADH-dependent reductase, we focused our investigation on the search of at least one of this kind of enzymes involved in the process.

A method was designed and developed to purify NADPH/NADH dependent enzymes, which are able to reduce selenite. Additionally, during the process we measured activities of enzymes that could be involved in reductase activity as catalase: CAT, glutamate dehydrogenate: GDH, alcohol dehydrogenate: ADH. Although CAT is not a NADPH/NADH dependent enzyme, we used it as control to demonstrate that selenite reduction is a NADPH/NADH dependent process.

For this investigation, we used the microorganism **GWE1**, isolated previously from a drying oven during the development of the AFOSR grant FA9550-06-1-0502. GWE1 (E1) is a thermophilic aerobic extremophile belonging to genus *Geobacillus* with the ability to reduce selenite, evidenced by the change of color of the culture from colorless to red (Figure 1).

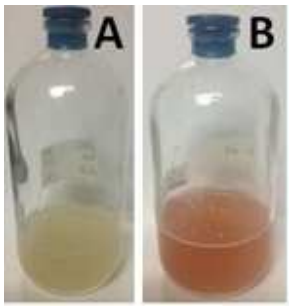

Figure 1. Change of color in cultures of GWE1 as result of reduction of selenite salt. (A): GWE1 without salt; (B): GWE1 after 22 h incubated with sodium selenite (Na_2SeO_3 x $5H_2O$).

In order to determine the characteristics of the reduced product of the reaction, we performed transmission electron micrograph and EDX analysis.

1.1 Transmission electron microscopy measurements (TEM) and Energy-Dispersive X-Ray Microanalysis (EDX).

To perform TEM, a sample was placed on a carbon coated copper grid and dried at room temperature. For size determination of nanoparticles, they were recovered from the microorganism. TEM measurements were performed on a Philips Tecnai 12 Bio Twin TEM microscope operating at 200 kV. EDX was conducted by using energy-dispersive X-ray microanalysis**.** To perform this, a scanning electron microscope (SEM) Jeol 5410 equipped with an energy dispersive X-ray spectrometer was used.

We visualized nanoparticles with a defined spherical shape. EDX analysis indicated that the elemental composition of nanoparticles was elemental selenium Se^0 (Figure 2).

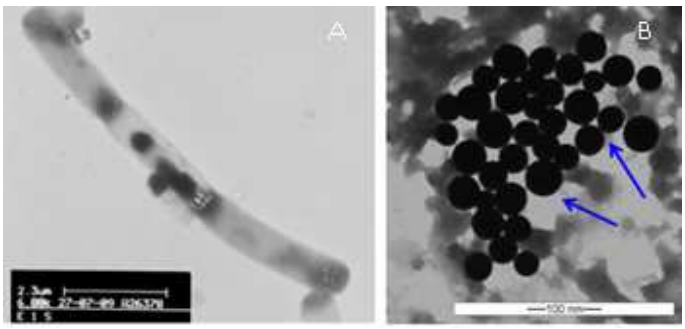

Figure 2. Selenium nanoparticles production by GWE1. (A): Transmission electron micrograph. Small black spots correspond to selenium nanoparticles accumulation within the microorganism body ; (B): Selenium nanoparticles produced by the microorganism on a copper grille.

1.2 Effect of different concentrations of NAD^+ in the reduction of selenite.

Previous experiments showed that the presence of whole microorganisms was not necessary for the reduction of selenite. Apparently a NADPH/NADH-dependent

reductase, extracted from this microorganism, mediates selenium nanoparticles synthesis under aerobic conditions.

To study the effect of different concentrations of NADH in the selenite reduction by GWE1, crude extract test tubes were prepared. The tubes contained crude extract from GWE1, a range of 0-10 mM NADH and 100 mM Na_2SeO_3 x $5H_2O$ and were incubated at 37°C for 12 h, without agitation. Control tubes contained only NADH and selenite. The reaction was followed by measuring the change in absorbance at 340 nm in a spectrophotometer. The presence of a red precipitate was indicative of selenite reduction. Figure 3 shows that NADH is involved in the selenite reduction by the GWE1 crude extract and it reaches saturation of concentration at around 3 mM of NADH.

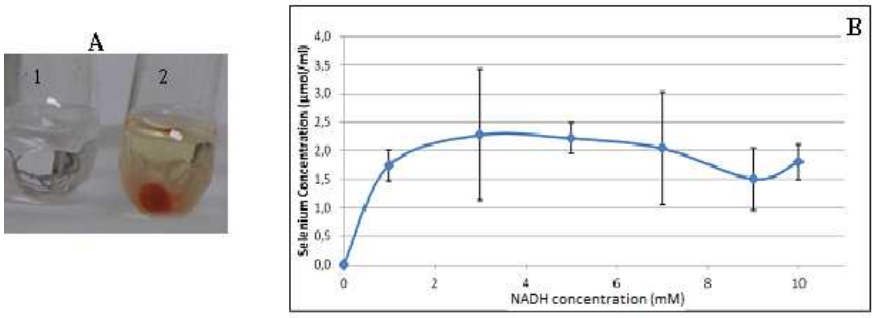

Figure 3. Enzymatic selenite reduction by GWE1 crude extract. (A): Reduction using crude extract of GWE1. Reddish color in tube 2 indicates selenium nanoparticles formation. Tube 1 is a control; (B): Selenium formation at different concentrations of NADH (1, 3, 5, 7, 9 and 10 mM).

Additionally, assays in the presence and absence of NADH were carried out for 2 commercial enzymes: glutamate dehydrogenase (GDH) from bovine liver and catalase from *Corynebacterium glutamicum* (CAT), both from Sigma, in order to study their possible involvement in the reduction of selenite.

1.3 Enzymatic assays.

1.3.1 GDH activity. To measure GDH activity, 100 µL enzyme, 10 mM NADH, 10 mM Na_2SeO_3 x $5H_2O$ and 10 mM glutamate were incubated at 25 ºC for 3 min (Table 1).

Table 1. Results of selenite reduction obtained measuring GDH activity. Symbology: presence of reagent (+); absence of reagent (-); reduction (R); no reduction (NR). The positive result is the experiment number 16 and is indicated with an arrow.

Nº	GDH	Selenite	NADH	Glutamate	Result
1	-	-	-	-	NR
2	+	-	-	-	NR
3	-	+	-	-	NR
4	+	+	-	-	NR
5	-	-	+	-	NR
6	+	-	+	-	NR
7	-	+	+	-	NR
8	+	+	+	-	NR
9	-	-	-	+	NR
10	+	-	-	+	NR
11	-	+	-	+	NR
12	+	+	-	+	NR
13	-	-	+	+	NR
14	+	-	+	+	NR
15	-	+	+	+	NR
→ **16**	**+**	**+**	**+**	**+**	**R**

Only in sample 16 when all components were present for the assay a reduction of selenite was observed**.**

1.3.2 CAT activity. To measure CAT activity, we used a catalase from *Corynebacterium glutamicum* (Sigma). The assay contained 50 µg/ml catalase, 10 mM NADH, 30 mM hydrogen peroxide and 30 mM Na_2SeO_3 x $5H_2O$ (Table 2).

Table 2. Results of selenite reduction obtained measuring CAT activity. Symbology: presence of reagent (+); absence of reagent (-); no reduction (NR); reduction (R).

Nº	CAT	Selenite	NADH	H_2O_2	Result
1	-	-	-	-	**NR**
2	+	-	-	-	**NR**
3	-	+	-	-	**NR**
4	+	+	-	-	**NR**
5	-	-	+	-	**NR**
6	+	-	+	-	**NR**
7	-	+	+	-	**NR**
8	+	+	+	-	**NR**
9	-	-	-	+	**NR**
10	+	-	-	+	**NR**
11	-	+	-	+	**NR**
12	+	+	-	+	**NR**
13	-	-	+	+	**NR**
14	+	-	+	+	**NR**
15	-	+	+	+	**NR**
16	+	+	+	+	**NR**

When similar experiment was performed but this time using CAT enzyme instead GDH enzyme none of the combinations assayed showed a reduction of selenite.

1.4 Enzyme purification.

Based on previous results, a protocol to purify a dehydrogenase enzyme from GWE1 crude extract involved in selenite reduction was designed. 20 g of cells grown as previously determined (AFOSR grant FA9550-06-1-0502), were suspended in 50 mM Tris-HCl pH 8.0 buffer containing 15 mM EDTA pH 8.0, lysozyme (1 mg/ml) and DNase I (10 µg/ml) and treated as described by Amenabar and Blamey, 2012. The crude extract was loaded into a column (Pharmacia, C 16/20) of Q-Sepharose Fast Flow (Pharmacia Biotech), equilibrated with 50 mM Tris-HCl pH 8.0 (buffer A). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 0 to 1 M NaCl in buffer A. Selenite reduction activity started to elute when 0.23 M NaCl was applied to the column. The fractions containing selenite reduction activity were combined and applied to a column (Pharmacia, C 10/10) containing DEAE-Sepharose Fast Flow (Pharmacia Biotech) equilibrated with 50 mM Tris-HCl pH 8.0 buffer. The elution was performed with a linear gradient of NaCl 0 to 1 M. Selenite reduction activity started to elute as 0.65 M NaCl. The fractions containing selenite reduction activity were combined and applied to a column Q-Hitrap (Pharmacia Biotech), equilibrated with 50 mM Tris-HCl pH 8.0 buffer. The fractions with selenite reduction activity started to elute when 0.11 M NaCl was applied to the column. The fractions were stored at 4 ºC. All columns were controlled by a Pharmacia FPLC system. To test the ability of fractions to perform selenite reduction, an assay was performed in each fraction.

1.4.1 Selenite reduction assay with fractions from purification process.

100 µL of each fraction obtained in the purification process was incubated separately with 10 mM NADH and 100 mM Na_2SeO_3 x $5H_2O$ at 37°C, for 12 h. The presence of a red precipitate was indicative of selenite reduction. Figure 4, 5 and 6 show that in each purification step, reduction of selenite was observed.

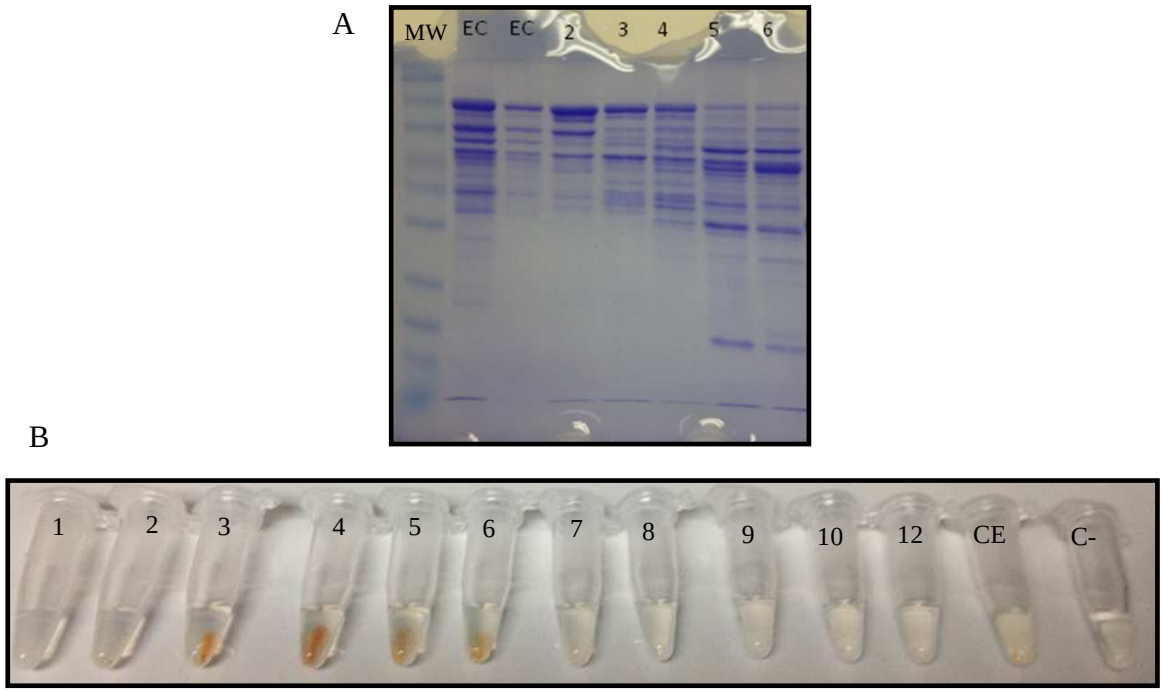

Figure 4. Selenite reduction assay for the fractions obtained from Q-Sephrose Fast Flow column. (A): SDS-Page of the 5 fractions with selenite reduction activity (2-6). MW: molecular weight. (B) Each tube corresponds to a fraction with activity. CE is the positive control (crude extract) and C- is the negative control without fraction. Tubes with red precipitate are positive for the selenite reduction (2-6).

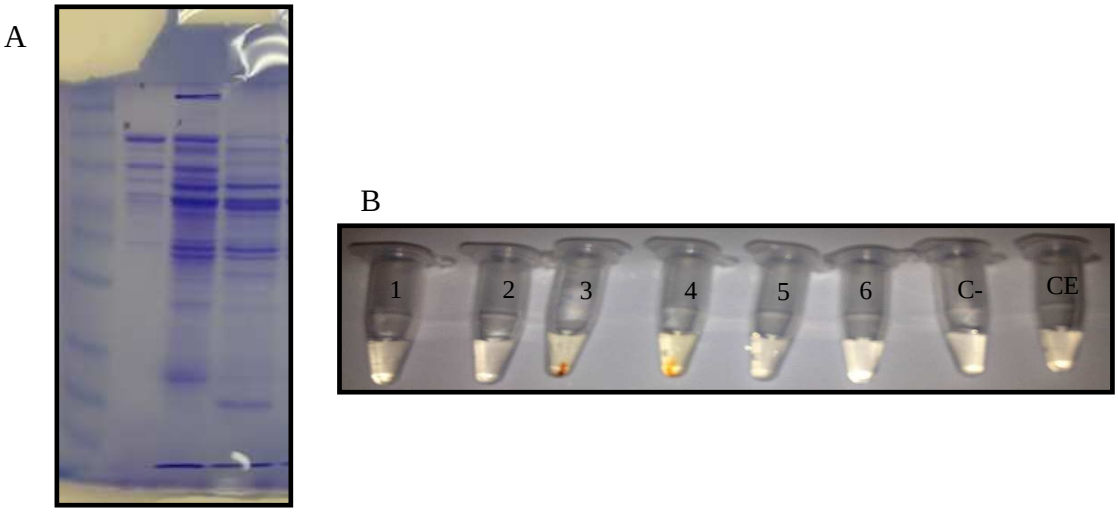

Figure 5. Selenite reduction assay for the fractions obtained from DEAE-Sepharose Fast Flow column. (A): SDS-Page of the 2 fractions with selenite reduction activity (3 and 4). MW: molecular weight marker. (B) Each tube with a number represents a fraction eluted from DEAE- Sepharose Fast Flow column. CE is the positive control (Crude extract) and C- is the negative control without fraction. Tubes with red precipitate are positive for the selenite reduction (3 and 4).

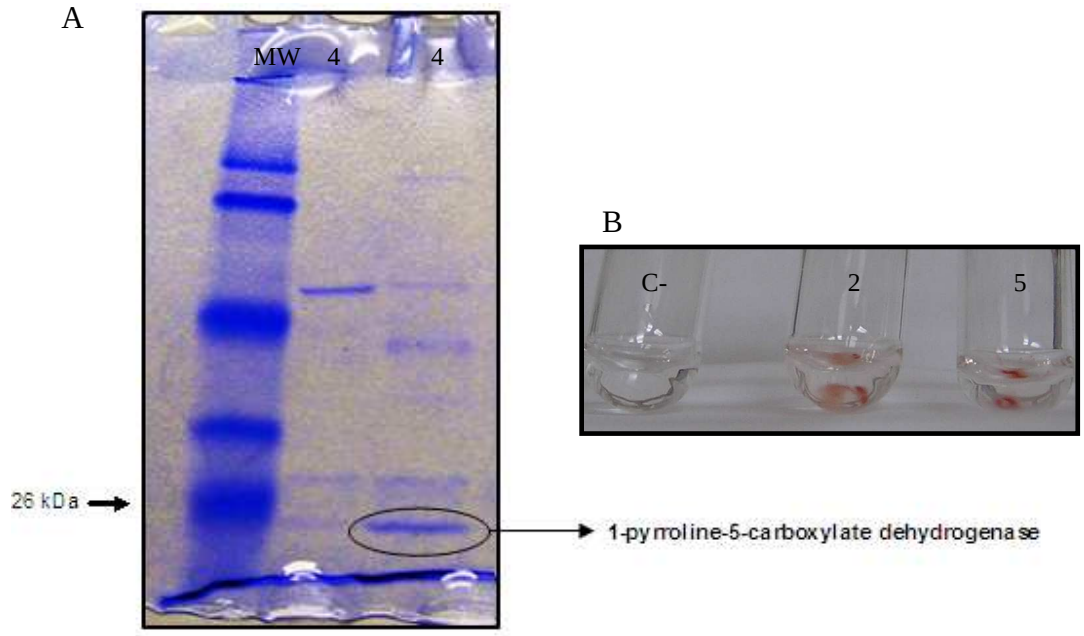

Figure 6. SDS-Page and selenite reduction assay for the fractions obtained from Q-Hitrap. (A) SDS-Page of the 2 fractions with activity. MW: molecular weight. The circle indicates the band with the protein of 26 kDa aprox. sent for identification. (B): Each tube with numbers represents a fraction with selenite reduction activity. CE is the positive control (Crude extract) and C- is the negative control without fraction. Tubes with red precipitate are positive for the selenite reduction.

We tested ADH activity in the fractions purified using the necessary controls and we obtained a very low activity of this enzyme. We sent the selected band of the SDS gel containing the enzyme, for identification to an specialized center in mass spectroscopy MALDI-TOF TOF technique. The results indicate that one of the enzymes implicated in this reduction process is a 1-pyrroline-5-carboxylate dehydrogenase (P5CDH). This enzyme is the second enzyme involved in proline metabolism, catalyzing the NAD+-dependent oxidation of L-glutamate-γ-semialdehyde to L-glutamate (Figure 6A).

SeNPs synthesized by P5CDH purified enzyme from *Geobacillus wiegelii,* GWE1 crude extract were observed by TEM and EDX. Histogram distribution of nanoparticles size was constructed using Statgraphics Centurion XV software. NPs sizes were obtained using NIS-Elements D 3.10 software. SeNPs synthesized by P5CDH were spherical (Fig 7A) of different sizes and smaller than the synthesized using the whole microorganism. SeNPs sizes ranged from 40-160 nm, where 20%

of them were of 100 nm of size (Fig. 7B). EDX analysis confirmed that the elemental composition of NPs was selenium Se^0(Figure 7C).

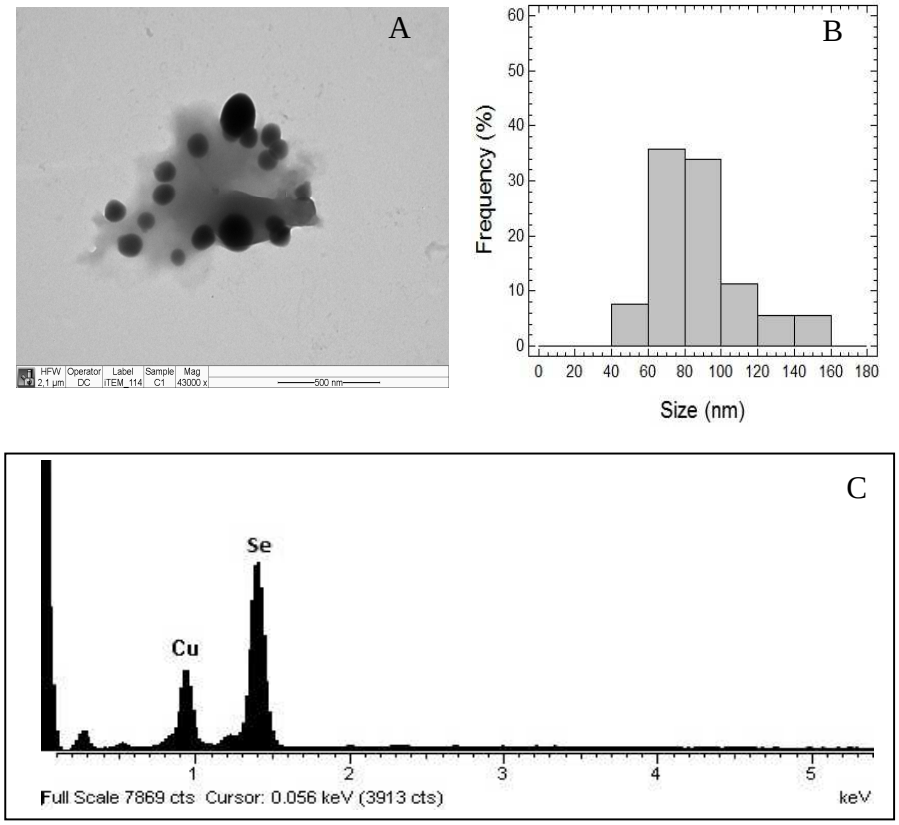

Figure 7. Selenium nanoparticles production for selenite reduction mediated by 1-pyrroline-5-carboxylate dehydrogenase enzyme purified from GWE1. (A): Transmission electron micrograph. Small spheres indicated with arrows, correspond to selenium nanoparticles. (B) Histogram showing the selenium nanoparticle size distribution. (C) EDX analysis of selenium nanoparticles. Arrows indicate selenium signals.

2. Objective: To study the effect of temperature and pH on the shape, size and stability of the nanoparticles formed in the enzyme-mediated process.

2.1 Effects of pH on selenium nanoparticles size and morphology.

With the purpose of controlling size and shape of SeNPs, different pH was used for the synthesis of NPs (4.0, 5.0, 6.0, 7.0, 8.0). We use 25 mM sodium acetate for pH 4.0 and 5.0, 25 mM MES for pH 6.0 and 7.0 and 25 mM EPPS for pH 8.0.

At pH 4.0 (Fig. 8A) 100% of SeNPs were less than 100 nm in size; at pH 5.0 (Fig. 8B) over 50% of their size was less than 100 nm; at pH 6.0 (Fig. 8C) and at pH 8.0 (Fig. 8E) over 90% of SeNPs size was less than 100 nm. Our experimental observations indicate that acid and basic pH favored the formation of NPs with sizes below 100 nm. At neutral pH (7.0) (fig. 8D) the NPs were bigger with a size around 120 nm. Only 20% of them were less than 100 nm. These results indicate that it is possible to modulate the size of SeNPs by changing the pH. Figure 8F, shows the media of selenium nanoparticles size distribution synthesized enzymatically and incubated at different pH (4.0, 5.0, 6.0, 7.0, 8.0).

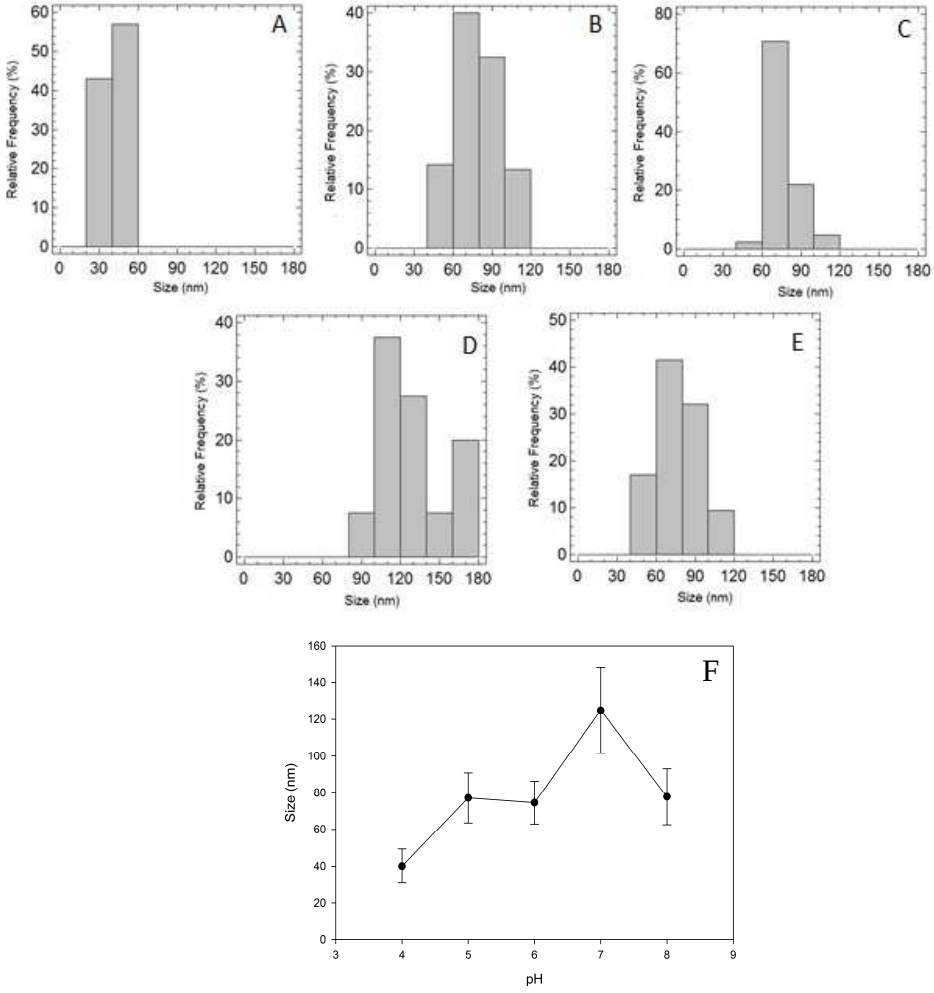

Figure 8. Histogram showing the enzymatically synthesized selenium nanoparticles size distribution at different pH. (A): pH 4.0; (B): pH 5.0; (C): pH 6.0; (D): pH 7.0; (E): pH 8.0; (F): Graph showing the selenium nanoparticles synthesized enzymatically and its growth media incubated at different pH (4.0, 5.0, 6.0, 7.0, 8.0).

2.1 Effects of temperature on selenium nanoparticles size and morphology.

After the synthesis, SeNPs were incubated at different temperatures for 1 h. When the temperature was 60 ºC (Fig. 9A) the NPs did not show a significant difference in size and shape compared to the control (Fig. 7B). At 80 °C a range size of nanoparticles from 20-160 nm was observed, with 35% of them over 100 nm (Fig. 9B). At 100 ºC (Fig. 9C), SeNPs had a size between 60-200 nm, with 70% of them over 100 nm.

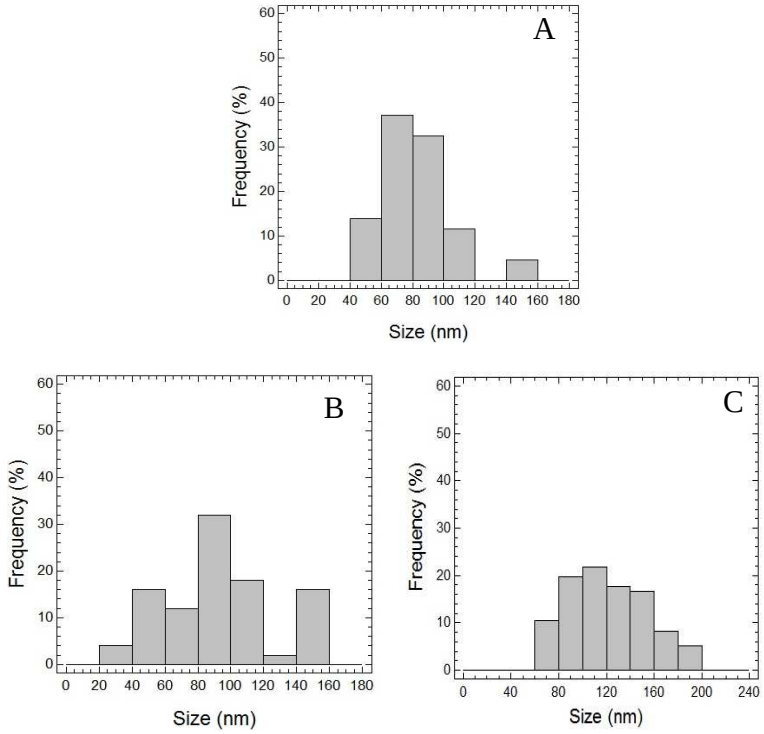

Figure 9. Effect of temperature on NPs synthesized enzymatically at 35 ºC in water. Histogram of NPs size incubated for 1h at: (a) 60 °C, (b) 80 °C and (c) 100 °C.

2.1 Effect of temperature on stability of selenium nanoparticles.

Selenium nanoparticles synthesized by the purified enzyme at 37 ºC and pH 6.0, were incubated at 60, 80, 100 ºC for 3 h. Then SeNPs were cooled in ice until room temperature. The changes in SeNPs experimented were observed by transmission electron microscopy.

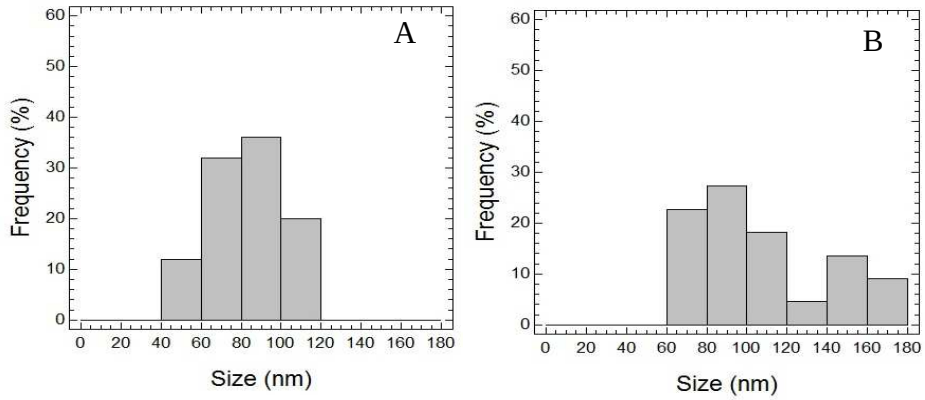

Figure 10. Effect of temperature on NPs enzymatically synthesized at 35 ºC. Histogram of NPs size incubated for 3h at: (a) 60 °C; (b); 80 °C.

For thermostability measurements, at 60 ºC after 3 h of incubation (Fig. 10A) the NPs did not show a significant difference compared with the control (Fig. 7B). At 80 °C after 3 h of incubation (Fig. 10B) NPs sizes range from 60-180 nm with 50% of them over 100 nm, showing a significant difference with the control (Fig. 7B), loosing homogeneity.

When the temperature was increased to 100ºC, after 2 hours of incubation the NPs formed nanorods structures with 3,4 µm of length and 125 nm of wide, size average (Fig. 11A-C) and large aggregates after 3 h of incubation at this temperature. These results demonstrate that at higher temperatures (over 60 ºC) NPs tend to form larger aggregates and rod structures. All these transformations occur in the presence of NADH and the purified enzyme. In conclusion, the experimental results presented indicate that biosynthesis of SeNPs is a redox enzymatic process mediated by some enzymes and NADH. Additionally, it is not

necessary the presence of the whole microorganism to generate nanoparticles. Size and shape of SeNPs can be modulated by pH and temperature.

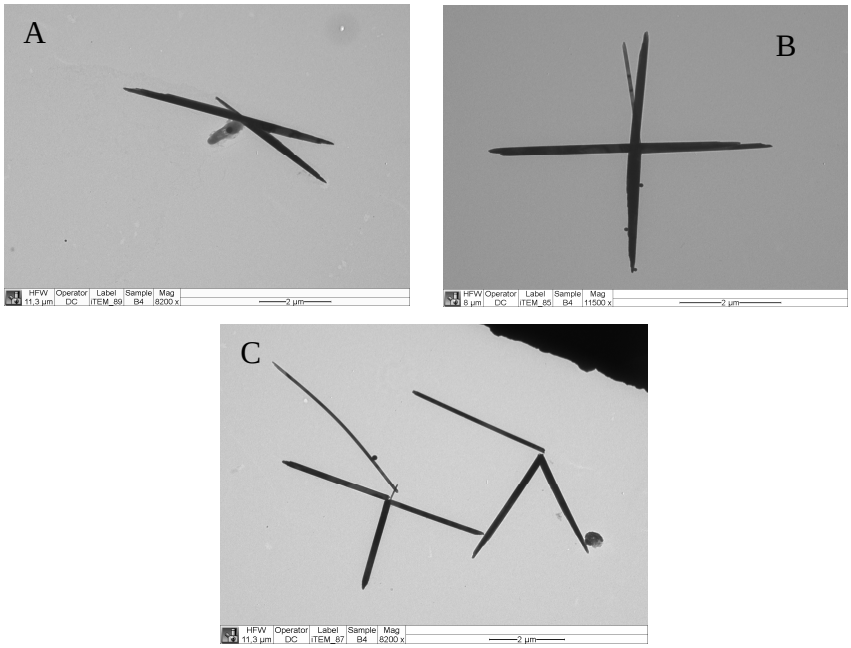

Figure 11. NPs incubated for 3 h at 100 °C. Figures (A), (B) and (C), shows larger structures formed called nanorods.

3. Additional work: Synthesis of gold nanoparticles.

Additionally, we produced gold nanoparticles using another microorganism belonging to genus *Geobacillus* called ID17 isolated from Deception Island, Antarctica. In this occasion it was possible to obtain colloidal gold nanoparticles using the whole microorganism and only the crude extract of this microorganism. The results and protocols obtained for this procedure were published in *Microbial Cell Factories* journal and the manuscript is attached to this report.

4. Summary of the Accomplished Work:

1 - Demonstration that GWE1 has the ability to produce selenium nanoparticles.
2 - Demonstration that enzymes are involved in the process of selenite reduction.
3 - Identification of the chemical mechanism of action of the enzymes.
4 - Demonstration that the biosynthesis of selenium and gold nanoparticles does not need the whole active metabolic machinery of the microorganism.
5 - Identification of one of the enzymes involved in selenium nanoparticles biosynthesis as 1-pyrroline-5-carboxylate dehydrogenase.
6 - Effect of pH on nanoparticles size and shape.
7 - Effect of Tº on nanoparticles size and shape.
8 - Effect of Tº on stability of nanoparticles.

5. Publications derived from this work.

1.- "Gold nanoparticles synthesized by *Geobacillus* sp. strain ID17 a thermophilic bacterium isolated from Deception Island, Antarctica". Daniela N. Correa-Llantén, Sebastian A. Muñoz-Ibacache, Miguel E. Castro, Patricio A. Muñoz and Jenny M. Blamey. *Microbial Cell Factories 2013, 12:75*

2.- "Enzyme Involvement in the Biosynthesis of Selenium Nanoparticles by *Geobacillus wiegelii* strain GWE1 Isolated from a Drying Oven", Daniela N. Correa-Llantén, Sebastián A. Muñoz-Ibacache, Mathilde Maire, Jenny M. Blamey submitted to *Word Academy of Science, Engineering and Technology*.

3.- "Synthesis of selenium nanoparticles by *Geobacillus wiegelii* strain GWE1 isolated from a drying oven", Muñoz-Ibacache, S. A., Correa-Llantén, D. N., Blamey J.M. submitted to *Biotechnology Reports.*

6. Presentations in Scientific Meetings.

1.- “Biosynthesis of Nanoparticles by two microorganisms belonging to genus *Geobacillus*“. Correa-Llantén, DN, Muñoz-Ibacache, SA, Muñoz, P, Blamey, JM. 12th International Meeting on Thermophiles; Regensburg, Germany. September 2013.

2.- “Identification of one enzyme Involved in selenium nanoparticles Biosynthesis in Geobacillus sp. strain GWE1 isolated from a drying oven”. Daniela N. Correa-Llantén, Sebastián A. Muñoz-Ibacache, Mathilde Maire, Jenny M. Blamey. ICBN: International Conference on Biotechnology and Nanotechnology; New York, USA, June 2014.

3.- “Biosynthesis of Nanoparticles by the Antarctic microorganism ID17”. Blamey, JM., Muñoz-Ibacache, SA., Muñoz, PA., Correa-Llantén, DN. SCAR 2014; Auckland, New Zealand. August 2014.

7. Personnel Involved in this project.

Principal investigator: Dra. Jenny Blamey.
Co-Researcher, Post-Doc.: Dra. Daniela Correa Llantén.
Undergraduate Student: Sebastián Muñoz Ibacache.